Biochemical and Biophysical Research Communications 446 (2014) 1073–1078

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The type II Ca^{2+}/calmodulin-dependent protein kinases are involved in the regulation of cell wall integrity and oxidative stress response in *Candida albicans*

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ARTICLE INFO

Article history:
Received 12 March 2014
Available online 21 March 2014

Keywords:
CaMKs
CWI
Oxidative stress
Candida albicans

ABSTRACT

The type II Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) are thought to play a vital role in cellular regulation in mammalian cells. Two genes *CMK1* and *CMK2* in the *Candida albicans* genome encode homologues of mammalian CaMKs. In this work, we constructed the *cmk1*Δ/Δ, the *cmk2*Δ/Δ and the *cmk1*Δ/Δ*cmk2*Δ/Δ mutants and found that CaMKs function in cell wall integrity (CWI) and cellular redox regulation. Loss of either *CMK1* or *CMK2*, or both resulted in increased expression of CWI-related genes under Calcofluor white (CFW) treatment. Besides, CaMKs are essential for the maintenance of cellular redox balance. Disruption of either *CMK1* or *CMK2*, or both not only led to a significant increase of intracellular ROS levels, but also led to a decrease of the mitochondrial membrane potential (MMP), suggesting the important roles that CaMKs play in the maintenance of the mitochondrial function.

1. Introduction

Fluctuations in intra cellular Ca^{2+} levels are known to initiate responses to environmental stimuli in a wide variety of cell types. One of the principal mediators of this Ca^{2+} signal in eukaryotic cells is calmodulin, a small Ca^{2+}-binding protein. Upon binding Ca^{2+}, calmodulin then changes its conformation, forming the Ca^{2+}–calmodulin complex that controls the activity of several key regulatory enzymes. In mammalian cells, this Ca^{2+}–calmodulin complex provides the essential ability to decode Ca^{2+} signals, acting to modulate the activities of a large number of protein kinases, the protein phosphatase calcineurin, nucleotide cyclases and phosphodiesterases, Ca^{2+} transporters and nitric oxide synthases [1,2].

In *Saccharomyces cerevisiae*, calmodulin is an essential protein, yet this essential function can still be performed by mutant proteins that do not bind Ca^{2+} [3]. The yeast Ca^{2+}–calmodulin complex is therefore dispensable for viability, even though it normally functions as an activator of a number of regulatory proteins. Notable Ca^{2+}–calmodulin targets are calcineurin and the type II Ca^{2+}–calmodulin dependent protein kinases (CaMKs) [4–6]. Calcineurin is important in cellular regulation in yeast. Its loss causes defects in the adaptation to endoplasmic reticulum stresses and osmostress [7–9].

At least two genes in *S. cerevisiae* encode homologues of mammalian CaMKs, which are responsible for decoding intracellular Ca^{2+} ion fluctuation in terms of a Ca^{2+}-mediated physiological response. They are *CMKI* and *CMK2* [4,10]. The deduced amino-acid sequences of Cmk1 and Cmk2 are 60% identical and 90% similar. Though gene-disruption analysis has revealed that single null mutants (*cmk1*Δ and *cmk2*Δ) and the double mutant (*cmk1*Δ*cmk2*Δ) grow normally at 17, 23, 30, and 37 °C and show no defects in meiosis or sporulation, Cmk2 plays an important role in suppressing tunicamycin-caused ROS accumulation [7]. Besides, as a putative substrate of Ste11, Cmk2 is essential for the maintenance of cell wall integrity (CWI). In fission yeast, Cmk2 is essential for oxidative stress response, and is identified as a new factor involved in oxidative stress-activated Sty1 MAP kinase response [11].

Candida albicans, the major human fungal pathogen, causes a range of disorders from mild infections to life-threatening diseases

Abbreviations: CaMKs, the type II Ca^{2+}/calmodulin-dependent protein kinases High affinity Ca^{2+} influx system; CWI, cell wall integrity; CFW, Calcofluor white; SC, synthetic complete; SD, synthetic drop-out; MMP, the mitochondrial membrane potential; 5-FOA, 5-fluoroorotic acid; DCFH-DA, 2′,7′-dichlorodihydro-fluorescein diacetate.

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http://dx.doi.org/10.1016/j.bbrc.2014.03.059
0006-291X/

[12,13]. Like other living cells, *C. albicans* cannot avoid the frequent challenge of oxidative stress by phagocytes when it survives and causes diseases in host [14]. Besides, *C. albicans* itself also generates various oxidative agents, such as reactive oxygen species (ROS), from the mitochondrial respiratory chain in the normal aerobic metabolism process. ROS, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, can damage many of the cellular components [15–17]. Therefore, regulation of the redox homeostasis is important for cellular functions [18,19]. To maintain intracellular redox homeostasis in *C. albicans*, a series of regulation mechanisms are involved, such as Ca^{2+}-mediated signaling pathways [20,21] and Hog1-mediated MAPK pathways [22,23]. However, the function of CaMKs in oxidative stress response or other aspects has not been explored in *C. albicans*.

Recently, we identified two genes encoding CaMKs in *C. albicans*, named *CMK1* and *CMK2*. In this work, we studied the roles of CaMKs in CWI and cellular redox regulation by constructing the *cmk1*Δ/Δ, the *cmk2*Δ/Δ and the *cmk1*Δ/Δ*cmk2*Δ/Δ mutants. We found that CaMKs play an important role in CWI. Loss of either *CMK1* or *CMK2*, or both resulted in the expression of CWI-related genes under CFW treatment. Besides, they are essential for the maintenance of the cellular redox balance. Disruption of either *CMK1* or *CMK2*, or both not only led to a significant increase of intracellular ROS levels, but also led to a decrease of the mitochondrial membrane potential (MMP), suggesting an important role that CaMKs play in the maintenance of the mitochondrial function.

2. Materials and methods

2.1. Strains, culture, and growth of C. albicans

C. albicans strains used in this study are derivatives of the wild-type strain BWP17 and listed in Table 1. Except where noted, *C. albicans* cells were grown at 30 °C in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) supplemented with 80 μg/ml uridine, or in synthetic complete (SC) medium (adding 80 μg/ml uridine), or in synthetic drop-out (SD) medium. Synthetic drop-out medium was used for the selection of transformants. SC medium supplemented with 0.1% 5-fluoroorotic acid (5-FOA; Lancaster, USA) was used to counter-select for *URA3*. Solid media contained 2% agar.

2.2. C. albicans strain construction

All deletion strains were generated in the BWP17 background. For the deletion of *CMK2* gene, the BWP17 strain was transformed with PCR products amplified from the plasmid pRS-ARG4Δ*Spe*I with the deletion primers *CMK2*-5DR (5′-CCATCCATAGATACATCAATT AGTTATTACCCACTTCTTGTATTAATCCCTTTACTTAACTTTCCCAGTCA CGACGTT-3′) and *CMK2*-3DR (5′-TTTCCTTCTTCATCTTCAGTA TAATTGGCTACTTTTTCTTTATTTGCAGTTGCTGCATGTGTGGAATTGT GAGCGGATA-3′), and the heterozygous mutant was confirmed by PCR with the detection primers *CMK2*-5det (5′-CGGTCATCAAAC AGTTATCA-3′) and *CMK2*-3det (5′-TCAACCAACATTCAGAGAAG-3′). The obtained heterozygous mutant was then transformed with PCR products amplified from the plasmid pDDB57 with the deletion primers, to generate the *cmk2*Δ/Δ null mutant. The *ura3* auxotrophs were obtained on SC agar plates containing 0.1% 5-FOA and 80 μg/ml uridine. In order to get the *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant, the *URA3* cassette amplified from the plasmid pDDB57 with the deletion primers *CMK1*-5DR (5′-ATACATATATAAATGTAGATTTTCCCCTAATT TTGGGTTTTCGCTTGTTCTCATCAACAATTTCCCAGTCACGACGTT-3′) and *CMK1*-3DR (5′-ATGTGATAAAGCTGGTGCTGACACCCCTCCTAC CTTTTGAAGAATATTTTTGATTTGATCGTGGAATTGTGAGCGGATA-3′) was used twice. First, the *cmk2*Δ/Δ strain was transformed with the *URA3* cassette, and the heterozygous mutant was confirmed by PCR with the detection primers *CMK1*-5det (5′-GTCATTATGGTACTCT-CAGG-3′) and *CMK1*-3det (5′-AGATCCATCTCTTGAAACTG-3′). After the heterozygous mutant was constructed, the *ura3* auxotrophs were obtained on SC agar plates containing 0.1% 5-FOA and 80 μg/ml uridine. Then the strain was transformed with the *URA3* cassette again, to generate the *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant. As for the construction of the *cmk1*Δ/Δ mutant, it shares the same way used for the construction of the *cmk2*Δ/Δ mutant.

2.3. CFW sensitivity

CFW sensitivity tests were set up in 96-well polystyrene flat-bottom microtitre plates (Denmark). Cell suspension (100 μL of 1×10^6 cells ml^{-1}) in YPD medium, containing the CFW ranging from 0 to 80 μg/ml, was added to wells of a microtitre plate. The plate was covered with its lid, sealed with parafilm and incubated at 30 °C for 24 h. OD_{600} (optical density at 600 nm) of each well was determined by using a microplate reader and the growth as a percentage of control (% of control) was calculated. Cells were also grown in solid YPD medium with indicated CFW concentration.

2.4. Oxidative-stress assays

Overnight cultures were refreshed in YPD medium and grown to log phase at 30 °C. Series of 10-fold dilutions were prepared in YPD, and approximately 10^6, 10^5, 10^4, 10^3, and 10^2 cells were

Table 1
Strains and plasmids in this study.

	Genotype	Source
Strains		
BWP17	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	Wilson
NKH1	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk1::ARG4/CMK1*	This study
NKH2	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk1::ARG4/cmk1:: dp1200*	This study
NKH3	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk1::ARG4/cmk1:: dpl200,CMK1*	This study
NKH4	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/CMK2*	This study
NKH5	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/cmk2:: dp1200*	This study
NKH6	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/cmk2:: dpl200,CMK2*	This study
NKH7	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/cmk2:: dp1200 cmk1:: dpl200/CMK1*	This study
NKH8	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/cmk2::dp1200 cmk1:: dpl200/cmk1::URA3-dpl200*	This study
Plasmids		
pRS-ArgΔSpeI	Ap^R *ARG4*	Dana Davis
pDDB57	Ap^R *URA3*	Dana Davis
pDDB78	Ap^R *TRP1 HIS1*	Dana Davis

spotted onto YPD plates supplemented with indicated H_2O_2 concentration.

2.5. Measurement of ROS levels

DCFH-DA (2′,7′-dichlorodihydro-fluorescein diacetate, Molecular Probes, USA) was used to measure the intracellular levels of reactive oxygen species (ROS) [19]. DCFH-DA crosses the cell membrane and is hydrolyzed by intracellular esterases to nonfluorescent dichlorofluorescin (DCFH). DCFH is converted to fluorescent 2′,7′-DCF (DCF) in the presence of intracellular reactive oxygen metabolites. Briefly, *C. albicans* cells cultured in YPD medium at the exponential growth phase were collected by centrifugation (3000*g*, 5 min, 4 °C) and washed three times with PBS. The cells were then resuspended in PBS (OD_{600} = 1.0). After being incubated with 20 μg/ml DCFH-DA at 30 °C for 30 min, the cells were exposed to H_2O_2 and incubated at 30 °C with constant shaking (200 rpm). At specified interval, fluorescence intensity was measured by flow cytometry, using a BD FACSCalibur flow cytometer (BD) with excitation at 485 nm and emission at 520 nm.

2.6. Measurement of MMP by flow cytometry

The change of MMP in *C. albicans* after H_2O_2 treatment was analyzed by using JC-1 Probes (5,5,6,6-tetrachloro-1,1,3,3-tetraethylimidacarbocyanine iodide, Sigma) [24]. JC-1 labels mitochondria with a high membrane potential red (JC-1 aggregates) as well as a low membrane potential green (JC-1 monomers). Briefly, *C. albicans* cells in exponential phase were treated with 5 mM H_2O_2 for 1 h at 30 °C. The fungal cells were washed three times and then resuspended in PBS (pH 7.4). JC-1 was added to the final concentration of 10 μg/ml and the mixture was incubated at 30 °C for 20 min. Then samples were immediately assessed for red and green staining by flow cytometry. A total of 10,000 gated events were analyzed per sample and a 488-nm filter was used for excitation of JC-1. Emission filters of 529 nm and 590 nm were used, respectively, to quantify the population of the cells with green and red fluorescence. Frequency plots were prepared for FL1 (green) and FL2 (red) to determine the percent of the population stained green and red. Results were expressed as percentage red and percentage green populations.

2.7. RNA isolation and real-time RT-PCR

RNA isolation and real-time RT-PCR were performed as described previously [16]. Triplicate experiments were performed with the Real-Time PCR System (Bio-Rad IQ5, USA). SYBR Green III (TaKaRa) was used to visualize and monitor the amplified products in real time. Gene-specific primers were designed according to the manufacturer's protocol. Primers for *ACT1* were 5′-GGTAGACCA AGACATCAAGG-3′ and 5′-CCGTGTTCAATTGGGTATCT-3′; Primers for *PGA13* were 5′-CTGGTACTGTAGTCTGTCCT-3′ and 5′-GTGACAAC GCTTCCTTCTTC-3′; primers for *CRH11* were 5′-TGATCGTGGTGGAT ATCATG-3′ and 5′- ATGGCCATT GGTGATTGTGG-3′; primers for *ECM331* were 5′-CTCATTACCTTCCTTTGCGA-3′ and 5′-AAC TATCGGT GACTGTTTGTA-3′; primers for *DFG5* were 5′-GTTGGCAAGATATAC TGGGA-3′ and 5′-CATCTCAAGTCAGTCACAGA-3′; primers for *GLR1* were 5′- GGAGATTTCGATTGGGCTAA-3′ and 5′-GACTTCAACTTCACC TTCAG-3′; primers for *TRR1* were 5′-TGGAGGATCTGAATTG ATGG-3′ and 5′-CACCAGTAGCAATGATAACG-3′. The change in fluorescence of SYBR Green III dye in every cycle was monitored by the Fit Point Method of LightCycler Software.

3. Results

3.1. CaMK mutants show no growth defect

In order to study the role(s) of CaMKs in *C. albicans*, growth curves of the wild-type strain, the *cmk1*Δ/Δ mutant, the *cmk2*Δ/Δ mutant and the *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant were measured in liquid YPD or SC media (adding uridine) at 30 or 37 °C. Growth in liquid medium was estimated as the absorbance at 600 nm (OD_{600}). We found that either at 30 °C or 37 °C, the *cmk1*Δ/Δ mutant, the *cmk2*Δ/Δ mutant and the *cmk1*Δ/Δ*cmk2*Δ/Δ mutant showed no growth defect in liquid YPD medium compared with the wild-type strain (Fig. 1A, C). Similar phenotype was also observed in liquid SC medium at 30 °C or 37 °C (Fig. 1B, D), indicating that loss of either *CMK1* or *CMK2*, or both has relatively little effect on the normal growth of *C. albicans*.

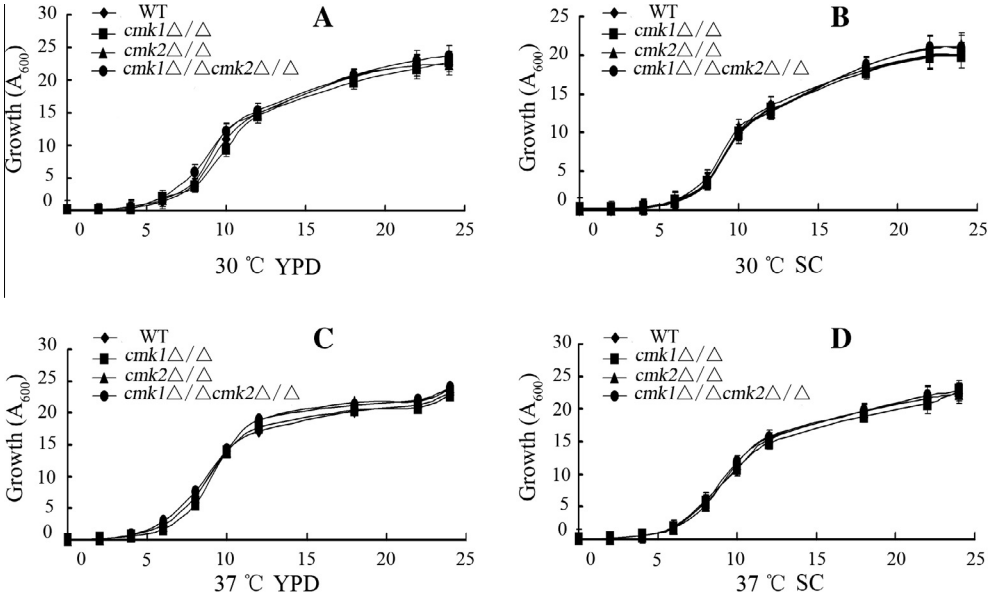

Fig. 1. Growth curves of WT and the CaMK mutants under different conditions. (A) Liquid YPD medium, 30 °C. (B) Liquid SC medium, 30 °C. (C) Liquid YPD medium, 37 °C. (D) Liquid SC medium, 37 °C. Results are shown as means ± SD of three independent experiments.

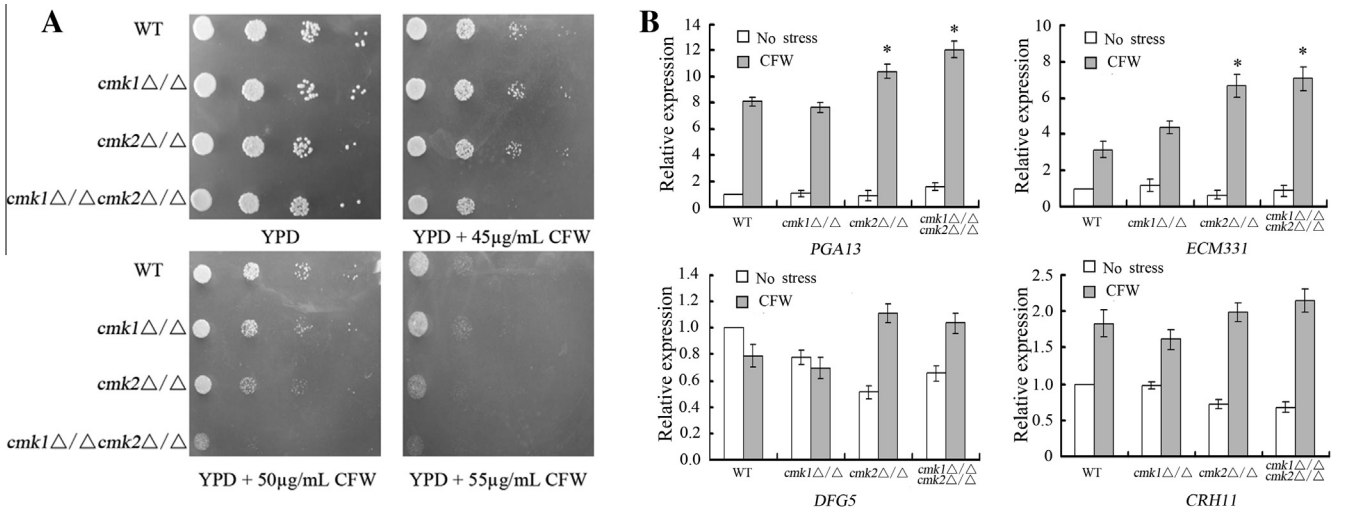

Fig. 2. The effect of CaMKs on CWI in *C. albicans*. (A) Sensitivity of the CaMK mutants to CFW. (B) Changes of expression of CWI-related genes in the CaMK mutants determined by real-time RT-PCR. Results are shown as means ± SD of three independent experiments. *Statistically significant differences between the mutants and the wild-type strain ($P < 0.01$).

3.2. CaMKs are essential for cell wall integrity (CWI)

STE11 encodes a mitogen activated protein kinase kinase kinase (MAPKKK) that activates conserved MAPK pathways controlling mating, high osmolarity glycerol (HOG), invasive growth and expression of CWI genes [25]. In *S. cerevisiae*, Cmk2, as a putative substrate of Ste11, plays an important role in CWI. To determine whether CaMks are required for CWI, we first tested the growth rates of the wild-type strain, the *cmk1*Δ/Δ mutant, the *cmk2*Δ/Δ mutant and the *cmk1*Δ/Δ*cmk2*Δ/Δ mutant in liquid YPD medium with indicated CFW concentration. Compared with the wild-type strain, the *cmk2*Δ/Δ mutant showed sensitivity to high CFW concentration while loss of *CMK1* had no obvious effect on the growth of the strain. However, disruption of both *CMK1* and *CMK2* led to increased sensitivity to CFW. 50 μg/ml CFW strongly blocked the growth of the mutant. Besides, the effect of CFW-induced growth defect of all the strains was dose-dependent (data not shown). To further confirm the role of CaMKs in CWI, spot assay experiments were also performed (Fig. 2A). Expectedly, under 45 μg/ml CFW treatment, both the *cmk1*Δ/Δ and the *cmk2*Δ/Δ mutants showed no growth defect compared with the wild-type strain, while the growth of the *cmk1*Δ/Δ*cmk2*Δ/Δ mutant was slightly inhibited. When the CFW concentration increased to 50 μg/ml, though the *cmk1*Δ/Δ mutant showed the same phenotype with the wild-type strain, the growth of the *cmk2*Δ/Δ and the *cmk1*Δ/Δ*cmk2*Δ/Δ mutants was both blocked and the *cmk1*Δ/Δ*cmk2*Δ/Δ mutant showed more sensitivity to CFW compared with the *cmk2*Δ/Δ mutant, raising the possibility of functional redundancy between Cmk1 and Cmk2. These results indicated that CaMKs are important for CWI under conditions of cell wall stress, and Cmk2 seems to be the main contributor in *C. albicans*.

3.3. CWI pathway was activated in CaMK mutants

Our previous work has demonstrated that CaMKs are essential for CWI in *C. albicans*. In order to further explore the role of CaMKs in maintaining CWI, quantitative RT-PCR was performed to analyze the expression of CWI-related genes *PGA13* (encoding a GPI-anchored cell wall protein involved in cell wall synthesis), *ECM331* (encoding a GPI-anchored cell wall protein), *DFG5* (encoding an N-linked mannoprotein of cell wall and membrane) and *CRH11* (encoding a GPI-anchored cell wall transglycosylase) [26–29]. We found that CFW treatment led to up-regulation of *PGA13, ECM331* and *CRH11* in all of the strains (Fig. 2B). The expression of *PGA13, ECM331*, *DFG5* and *CRH11* in *cmk2*Δ/Δ and *cmk1*Δ/Δ*cmk2*Δ/Δ cells was remarkably up-regulated compared with the wild-type strain, while no obvious differences were observed in *cmk1*Δ/Δ cells. The expression of CWI-related genes in *cmk1*Δ/Δ*cmk2*Δ/Δ cells was even more than that in *cmk2*Δ/Δ cells. Therefore, we concluded that there existed functional redundancy between Cmk1 and Cmk2 in maintaining CWI in *C. albicans*. Loss of either *CMK1* or *CMK2*, or both may activate an unknown mechanism, thus resulting in the expression of CWI-related genes under CFW treatment.

3.4. CaMKs are essential for oxidative stress response

Oxidative stress is one of the most important challenges that *C. albicans* has to face during its infection. To explore the role of CaMKs in response to oxidative stress, spot assay experiments were performed. We found that disruption of *CMK1* had no obvious effect on the growth. The *cmk2*Δ/Δ cells showed slight growth defect, and the growth of the *cmk1*Δ/Δ*cmk2*Δ/Δ cells was strongly blocked under H_2O_2 treatment (Fig. 3A), indicating that CaMKs play an important role in response to oxidative stress in *C. albicas*.

As a free radical generator, H_2O_2 is known to have a deleterious effect on cell growth. One of the major mechanisms is that when exposed to H_2O_2, intracellular reactive oxygen species (ROS) increase remarkably. To examine whether exposure to H_2O_2 causes an increased intracellular ROS level in the CaMK mutants, fluorescent dye DCFH-DA was used to determine ROS generation of the cells. Expectedly, an increase of intracellular ROS level was observed in all of the strains upon H_2O_2 treatment. However, the increases were even stronger in the CaM kinase II mutants, especially in the *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant. The percentages of ROS-accumulated CaM kinase II mutant cells were 2.41% (*cmk1*Δ/Δ), 4.49% (*cmk2*Δ/Δ) and 7.46% (*cmk1*Δ/Δ*cmk2*Δ/Δ), respectively (Fig. 3B), while only 1.35% of the wild-type cells showed ROS accumulation. These results suggested that the remarkable accumulation of intracellular ROS partially leads to the hypersensitivity of the CaMK mutants to H_2O_2.

3.5. CaMKs mutants show decreased mitochondrial membrane potential

Mitochondria are the main generator of intracellular ROS. We therefore investigated the effects of CaMKs on mitochondria

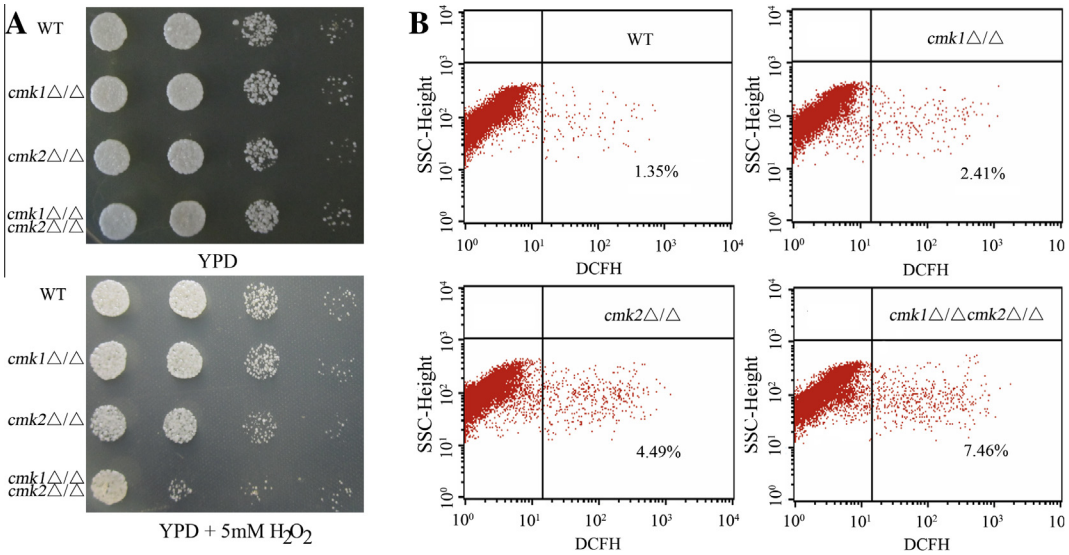

Fig. 3. Sensitivity of the CaMK mutants to H_2O_2 treatment (A) and changes of the levels of ROS in different strains of *C. albicans* (B). ROS levels was measured using DCFH-DA. The percent of DCF-positive cells was indicated.

function by MMP, the direct indicator of mitochondrial function, of the CaMK mutants. 6.48% of the wild-type strain cells showed a decrease of the MMP, while the percentages of CaMK mutant cells that showed a decrease of the MMP were 7.97% (*cmk1*Δ/Δ), 14.01% (*cmk2*Δ/Δ) and 29.18% (*cmk1*Δ/Δ*cmk2*Δ/Δ), respectively (Fig. 4A), suggesting that CaMKs are essential for the maintenance of the mitochondrial function.

3.6. CaMKs mediate oxidative stress response

Since the CaMKs play an important role in the response to oxidative stress, real-time RT-PCR was performed to investigated the expression of some important redox-related genes, including *GLR1* (encoding glutathione reductase gene) and *TRR1* (encoding thioredoxin reductase gene). We found that H_2O_2 treatment induced *GLR1* and *TRR1* expression in all of the strains (Fig. 4B). However, *GLR1* and *TRR1* expression was down-regulated in *cmk1*Δ/Δ mutant while in *cmk2*Δ/Δ cells it showed the opposite result. Cmk1 and Cmk2 provided additive functions based on a greater block of the expression of *GLR1* and *TRR1* in *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant than either single mutant, suggesting that CaMKs are involved in the regulation of redox homeostasis of *C. albicans*.

4. Discussion

In this work, we constructed the CaMK mutants and identified the roles that CaMKs played in *C. albicans*. We found that the *cmk1*Δ/Δ, *cmk2*Δ/Δ and *cmk1*Δ/Δ*cmk2*Δ/Δ mutants showed no growth defect in liquid YPD or SC medium at 30 °C or 37 °C, indicating that CaMKs are not essential for *C. albicans* growth under normal conditions. However, these kinases play important roles

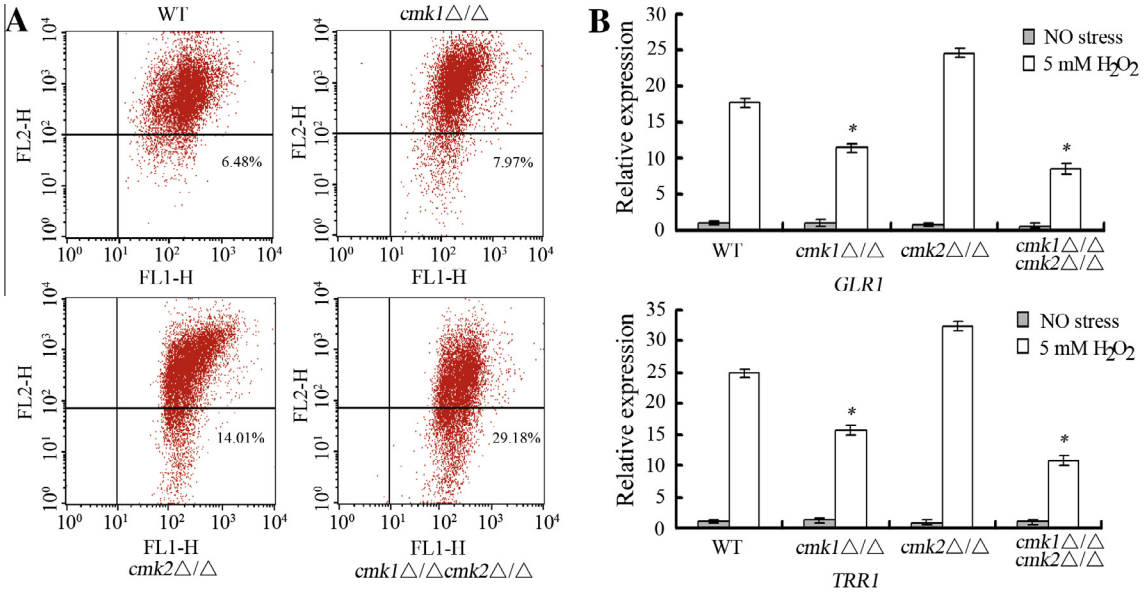

Fig. 4. (A) The mitochondrial membrane potential in different strains of *C. albicans*. JC-1 labels mitochondria with a high membrane potential red (JC-1 aggregates) and mitochondria with a low membrane potential green (JC-1 monomers). Cells staining red appear in the upper right quadrant (UR); green-stained cells appear in the lower right quadrant (LR). The percent of cells with decreased MMP was demonstrated. (B) Changes of expression of oxidative stress response genes in CaMK mutants determined by real-time RT-PCR. Results are shown as means ± SD of three independent experiments. *Statistically significant differences between the mutants and the wild-type strain ($P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in CWI. Compared with the wild-type strain, though no obvious differences were observed in *cmk1*Δ/Δ mutant under CFW treatment, the growth of the *cmk2*Δ/Δ mutant was slightly inhibited. Besides, Cmk1 and Cmk2 provided additive functions based on a greater block in *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant than either single mutant. We also found that CWI pathway was activated in CaMK mutants. The expression of *PGA13*, *ECM331*, *DFG5* and *CRH11* in *cmk2*Δ/Δ and *cmk1*Δ/Δ*cmk2*Δ/Δ cells was remarkably up-regulated compared with the wild-type strain, while no obvious differences were observed in *cmk1*Δ/Δ cells. Besides, the expression of CWI-related genes in *cmk1*Δ/Δ*cmk2*Δ/Δ cells was more increased than that in *cmk2*Δ/Δ cells, which were consistent with the results described above. Since in *S. cerevisiae*, Cmk2, as a putative substrate of Ste11, plays an important role in CWI [25], we concluded that loss of either *CMK1* or *CMK2*, or both may activate an unknown mechanism in the CWI pathway, thus resulting in the expression of CWI-related genes under CFW treatment.

C. albicans cannot avoid the frequent challenge of oxidative stress by phagocytes when it survives and causes diseases in host [14]. *C. albicans* has evolved a series of ways to respond to the environmental changes. In this study, we explored the roles of CaMKs playing in cellular redox regulation. However, disruption of *CMK1* had no obvious effect on the growth under H_2O_2 treatment. Besides, though loss of *CMK2* led to a slight growth defect in *C. albicans*, the *cmk2*Δ/Δ mutant was not so susceptible to H_2O_2 just as that in fission yeast [11]. Only the *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant showed hypersensitivity to H_2O_2 in this pathogen. Besides, flow cytometry-based analysis revealed that CaMK mutants showed an increased intracellular ROS levels compared with the wild-type strain when exposed to H_2O_2, which partially explained why the mutants are so susceptible to oxidative stress.

Mitochondrial respiratory chain is the main source of intracellular ROS and MMP is an important parameter on the redox status of mitochondria. Sophisticated regulation mechanisms exist between intracellular ROS and MMP [30]. Since levels of intracellular ROS were affected by loss of either *CMK1* or *CMK2*, or both, we also investigated the changes of MMP in the CaMK mutants. We found that CaMKs are essential for the maintenance of the mitochondrial function. The MMP was decreased in the mutants, especially in the *cmk1*Δ/Δ*cmk2*Δ/Δ cells. We proposed that this decrease is associated with intracellular ROS accumulation in the cells.

Deletion of either *CMK1* or *CMK2*, or both led to a significant increase of intracellular ROS levels, indicating that CaMKs play an important role in the maintenance of intracellular redox balance. We postulated that some reductases might be down-regulated in the CaMK mutants. Unexpectedly, loss of *CMK2* led to the up-regulation of *GLR1* and *TRR1* while loss of *CMK1* led to the opposite result. Besides, the expression of *GLR1* and *TRR1* showed a greater block in *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant than either single mutant, indicating that Cmk1 and Cmk2 are both required for the oxidative stress response and provide additive functions in the regulation of redox homeostasis in *C. albicans*.

Acknowledgments

We thank Dana Davis (University of Minnesota, USA) for generously providing strains and plasmids. We thank Xinglong Zhou for flow cytometry assays. We also thank reviewers for critical reading and helpful suggestions. This work was supported by National Natural Science Foundation of China (No. 81171541) and Natural Science Foundation of Tianjin (13JCYBJC20700).

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